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Identification of a Gene Cluster Enabling *Lactobacillus casei* BL23 To Utilize *myo*-Inositol▽†

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Genome analysis of *Lactobacillus casei* BL23 revealed that, compared to *L. casei* ATCC 334, it carries a 12.8-kb DNA insertion containing genes involved in the catabolism of the cyclic polyol *myo*-inositol (MI). Indeed, *L. casei* ATCC 334 does not ferment MI, whereas strain BL23 is able to utilize this carbon source. The inserted DNA consists of an *iolR* gene encoding a DeoR family transcriptional repressor and a divergently transcribed *iolTABCDG1G2EJK* operon, encoding a complete MI catabolic pathway, in which the *iolK* gene probably codes for a malonate semialdehyde decarboxylase. The presence of *iolK* suggests that *L. casei* has two alternative pathways for the metabolism of malonic semialdehyde: (i) the classical MI catabolic pathway in which IolA (malonate semialdehyde dehydrogenase) catalyzes the formation of acetyl-coenzyme A from malonic semialdehyde and (ii) the conversion of malonic semialdehyde to acetaldehyde catalyzed by the product of *iolK*. The function of the *iol* genes was verified by the disruption of *iolA*, *iolT*, and *iolD*, which provided MI-negative strains. By contrast, the disruption of *iolK* resulted in a strain with no obvious defect in MI utilization. Transcriptional analyses conducted with different mutant strains showed that the *iolTABCDG1G2EJK* cluster is regulated by substrate-specific induction mediated by the inactivation of the transcriptional repressor IolR and by carbon catabolite repression mediated by the catabolite control protein A (CcpA). This is the first example of an operon for MI utilization in lactic acid bacteria and illustrates the versatility of carbohydrate utilization in *L. casei* BL23.

myo-Inositol (MI) is the most abundant stereoisomer of inositol (1,2,3,4,5,6-cyclohexanehexol). It is common in soils, and it is a constituent of phytic acid (inositol hexaphosphate), which in the form of various salts (phytates) provides a major phosphate storage molecule in plant seeds. Several microorganisms, mostly inhabitants of soil, can utilize MI as a carbon source. For bacteria, the MI catabolic pathway has been elucidated for *Enterobacter aerogenes* (formerly *Aerobacter aerogenes*) (3). MI is important for the establishment of symbiosis in legume nodules. The metabolism of MI in *Sinorhizobium fredii* and *Rhizobium leguminosarum* is required for efficient nitrogen fixation and nodulation of soybeans (9, 15). MI dehydrogenase genes (e.g., *iolG*) and inosose dehydratase genes (*iolE*) have been characterized in *S. fredii* and *Sinorhizobium meliloti* (10, 15, 36), and *iolA* and *iolDEB* genes have been studied in *R. leguminosarum* (9). An MI utilization gene cluster has also been described for *Clostridium perfringens*, which was induced by MI via the IolR regulator (16), and recently, transcriptome analysis permitted the identification of an *iol* cluster which allowed the rapid growth of *Corynebacterium glutamicum* on MI (19). However, the first MI catabolic gene cluster was characterized in *Bacillus subtilis*, and detailed molecular data are available only for this bacterium. The *B. subtilis iolRS* and *iolABCDEFGHIJ* divergon and the *iolT* gene are involved in MI catabolism (39, 40). These genes are regulated by the product of *iolR*, a repressor which binds to the promoter regions and blocks transcription of the three transcriptional units in the absence of MI (41). In the presence of MI, a molecule derived from its metabolism binds IolR, thereby preventing its interaction with the DNA. The *B. subtilis iol* regulon is also subject to carbon catabolite repression (CCR) mediated by the catabolite control protein A (CcpA) and its corepressor, the HPr protein of the phosphoenolpyruvate:sugar phosphotransferase system (PTS) phosphorylated at serine residue 46 (11, 24).

MI utilization is not a common feature among lactic acid bacteria (LAB), which are defined as a group of microaerophilic, gram-positive organisms that ferment carbohydrates to produce primarily lactic acid. Taxonomically, LAB are classified within the order *Lactobacillales*, which includes, among others, the genus *Lactobacillus*. Although many lactobacilli can grow on plant-derived material, ferment hexitols, or possess phytase activity able to release phosphate from phytates (21, 28), there is no report on MI catabolism for this bacterial group. Furthermore, no genetic locus involved in MI catabolism has been characterized for members of the *Lactobacillales*. For example, the genome sequence of *Lactobacillus plantarum* reveals only the presence of an incomplete MI catabolic pathway (18), whereas genes related to MI catabolism are absent from the other 45 lactobacillales whose genomes have been partially or completely sequenced (22), including *Lactobacillus*, *Lactococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, and *Enterococcus* strains. Nevertheless, we report here the character-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics[a]	Source or reference[b]
Strains		
Lactobacillus casei		
BL23	Wild type, inositol$^+$, genome sequenced at Université de Caen, CNRS, INRA, and CSIC	B. Chassy, University of Illinois
BL121	BL23 *ptsH1* (HPrSer46Ala)	32
BL190	BL23 Δ*ccpA*	8
BL254	BL23 *iolT*::pRV300 Ery^r	This work
BL255	BL23 *iolR*::pRV300 Ery^r	This work
BL256	BL23 *ptsH1 iolR*::pRV300 Ery^r	This work
BL264	BL23 *iolD*::pRV300 Ery^r	This work
BL265	BL23 *iolK*::pRV300 Ery^r	This work
BL276	BL23 *iolA* (frameshift at SpeI site)	This work
ATCC 334	Type strain, inositol$^-$, genome sequenced at JGI	ATCC
ATCC 393	Wild type, inositol$^-$, reclassified as *Lactobacillus zeae*	ATCC
ATCC 4646	Wild type, inositol$^+$	ATCC
ATCC 11578	Wild type, inositol$^+$	ATCC
CECT 4040	Wild type, inositol$^-$	CECT
CECT 4043	Wild type, inositol$^-$	CECT
64H	Wild type, inositol$^-$	12
61BG	Wild type, inositol$^+$	12
Lactobacillus plantarum		
NCIMB8826	Wild type, inositol$^-$, genome sequenced at WCFS	NCIMB
ATCC 14917	Type strain, inositol$^-$	ATCC
Plasmids		
pRV300	Insertional vector for *Lactobacillus*; Amp^r Ery^r	20
pMOS*Blue*	Cloning vector; Amp^r	Amersham Biosciences
pRViolT	pRV300 with a 0.6-kb *iolT* fragment cloned at KpnI and EcoRI sites	This work
pRViolR	pRV300 with a 0.5-kb *iolR* fragment cloned at KpnI and EcoRI sites	This work
pRViolD	pRV300 with a 0.6-kb *iolD* fragment cloned at SmaI site	This work
pRViolK	pRV300 with a 0.3-kb *iolK* fragment cloned at SmaI site	This work
pRViolASpeI	pRV300 with a 2.2-kb EcoRI-HindIII fragment from pMiolTDSpeI	This work
pMiolTD	pMOS*Blue* with a 5.8-kb fragment from *iolT* to *iolD* cloned at EcoRV site	This work
pMiolTDSpeI	pMiolTD with a frameshift at SpeI site in *iolA*	This work

[a] CNRS, Centre National de la Recherche Scientifique; INRA, Institut National de la Recherche Agronomique; CSIC, Consejo Superior de Investigaciones Científicas; JGI, Joint Genome Institute; WCFS, Wageningen Centre for Food Sciences. inositol$^+$, able to produce acid from *myo*-inositol; inositol$^-$, unable to produce acid from *myo*-inositol.

[b] CECT, Colección Española de Cultivos Tipo; NCIMB, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland.

ization of an operon of *Lactobacillus casei* strain BL23 involved in MI catabolism.

MATERIALS AND METHODS

Strains and growth conditions. *Lactobacillus* strains used in this study are listed in Table 1. They were grown in MRS broth (Oxoid) or MRS fermentation medium (Scharlau Chemie S.A., Barcelona, Spain) plus 0.5% (wt/vol) of different sugars at 37°C under static conditions. When required, growth was performed under agitation at 200 rpm in 50-ml Erlenmeyer flasks containing 10 ml of medium. *Escherichia coli* DH5α was used as a cloning host and was grown in LB medium at 37°C under agitation. The antibiotics used were 100 μg/ml ampicillin for *E. coli* and 5 μg/ml erythromycin for *L. casei*.

Construction of strains. To construct different *L. casei* mutants, internal fragments of *iolR*, *iolT*, *iolD*, and *iolK* were amplified by PCR and cloned in the integrative vector pRV300 (20). Oligonucleotide pairs were as follows (with restriction sites in italics): 5′-TTC*GGTACC*TTCAGAGTTTGA/5′-AAC*GAATTC*GATGCTGGTACTG for *iolR*, 5′-GGC*GAATTC*TCGGTGATCGTG/5′-AAT*GGTACC*AACGACAGACGT for *iolT*, 5′-GAAGCGTGTTCATGTTGTTA/5′-CAATAACTGCCTCAGTTTGT for *iolD*, and 5′-CGATCACCTGAAGAGATTAA/5′-GATCATCATTGAATGAAACG for *iolK*. The PCR products corresponding to *iolR* and *iolT* fragments were digested with EcoRI/KpnI and cloned into pRV300 digested with the same enzymes. *iolD* and *iolK* fragments were directly cloned into SmaI-digested pRV300. PCR amplifications were carried out with Platinum *Pfx* polymerase (Invitrogen). The resulting plasmids (pRViolR, pRViolT, pRViolD, and pRViolK) were used to transform *L. casei* BL23, and single-crossover integrants were selected by resistance to erythromycin and confirmed by PCR. To construct an *iolA* mutant, a 5.8-kb DNA fragment expanding from the 5′ half of *iolT* to the 3′ half of *iolD* was amplified with oligonucleotides 5′-GGCGAATTCTCGGTGATCGTG and 5′-CAATAACTGCCTCAGTTTGT by using the Expand-High Fidelity kit (Roche) and cloned into EcoRV-digested pMOS*Blue* (Amersham). The obtained plasmid (pMiolTD) was partially digested with SpeI, treated with the Klenow fragment of DNA polymerase I, ligated, and transformed. One plasmid-containing clone in which a frameshift was introduced at the SpeI site in *iolA* (pMiolTDSpeI) was selected. This plasmid was digested with EcoRI-HindIII, and a 2.2-kb fragment carrying the mutated *iolA* gene was cloned into pRV300 digested with the same enzymes, giving pRViolASpeI. *L. casei* was transformed with this plasmid, and one erythromycin-resistant clone carrying the plasmid integrated by a single-crossover event was grown in MRS without erythromycin for 200 generations. Cells were plated on MRS and replica plated on MRS plus erythromycin. Antibiotic-sensitive clones were isolated, and among them, one was selected (the BL276 strain) in which a second recombination event led to the excision of the plasmid leaving a mutated *iolA* copy, as was confirmed by sequence analysis of appropriate PCR products. Standard methods were used for cloning in *E. coli*. Restriction enzymes, Klenow enzyme, and T4 DNA ligase were purchased from New England Biolabs. *Taq* DNA polymerase for PCR screening was from Biotools (B & M Labs, Madrid, Spain). Plasmids were isolated with a GFX micro plasmid prep kit (Amersham). DNA from *L. casei* was isolated with an UltraClean microbial DNA isolation kit (MoBio Laboratories, Solana Beach, CA). *L. casei* BL23 was transformed by electroporation with a Gene Pulser apparatus (Bio-Rad) as previously described (27).

Northern blots. For RNA isolation, strains were grown in 10 ml of MRS fermentation medium with different sugars under agitation until an optical den-

sity at 550 nm of 0.8 to 0.9 was reached. Cells pregrown in MRS fermentation medium plus either ribose or MI were used as inocula (starting optical density at 550 nm was 0.05). Cells were collected by centrifugation, washed with 10 ml of 50 mM EDTA, pH 8, and resuspended in 1 ml of TRIzol (Gibco). One gram of 0.1-mm glass beads was added, and cells were broken with a MiniBeadbeater apparatus (Biospec Products, Bartlesville, OK). RNA was isolated as described by the manufacturers of TRIzol, and 5-μg aliquots were separated on formaldehyde-agarose (1%) gels in a buffer with a pH of 7 and containing 40 mM MOPS (morpholinepropanesulfonic acid), 10 mM acetate, and 1 mM EDTA. RNA was transferred to Hybond N+ membranes (Amersham) and hybridized with digoxigenin-labeled probes. Probes were synthesized by PCR using the same oligonucleotides designed for gene disruption and the PCR-digoxigenin labeling mix from Roche. Hybridization, washing, and detection were performed as recommended by the supplier with the CDP-Star (Roche) chemiluminescent reagent.

MI dehydrogenase assays. Cells of strain BL255 were grown in the presence of either ribose, glucose, ribose plus MI, or ribose plus MI plus glucose. The preparation of crude extracts and enzyme assays were carried out as previously described (6), except that 500-μl instead of 100-μl volumes of crude extracts were used for each assay.

Nucleotide composition similarity and phylogenetic analysis. In order to determine the nucleotide composition similarity between the *iol* gene cluster and the complete *L. casei* ATCC 334 genome sequence, dinucleotide composition differences were estimated by comparing their δ* values calculated using the δρ-web server (31). Briefly, the δ* value of the *iol* gene cluster was compared to the δ* values of fragments of the host genomes that were equal in size. A high compositional dissimilarity between the gene cluster and the host genome is reflected by a large percentage of genomic fragments with lower δ* values than the δ* value for the cluster.

Bacterial genes encoding homologues of IolD, IolE, IolG, and IolK were retrieved from whole genomes (NCBI repository, June 2006) by using BLASTP and TBLASTN (1, 2), and the genes carried by *L. casei* were used as query sequences. The data sets were refined by excluding redundant sequences, sequences that shared significant similarities in fewer than 80% of their coding sequences, and sequences with expected values (E) of more than 10^{-102} (*iolD*), 10^{-9} (*iolE*, *iolG1*, and *iolG2*), and 10^{-1} (*iolK*). Some sequences were modified as follows. A possible frameshift in the putative *iolE* sequence of *Bacillus anthracis* strain Sterne (GenBank accession number AAT54648) was corrected by inserting one A between positions 2339181 and 2339186 and moving the start site to position 2339008 in the genome nucleotide sequence (accession number NC_005945). The start site of *B. subtilis iolD* was moved to position 4080027 in the sequence corresponding to accession number NC_000964. The start site of the *iolE* homologue RHE_PF00446 of *Rhizobium etli* (labeled as retli1 [see Fig. 3]; YP_473063) was moved to position 499306 in the sequence corresponding to accession number NC_007766. The start site of the *iolG* homologue RHE_CH01209 of *Rhizobium etli* (labeled as retli1; YP_468741) was moved to position 1261472 in the sequence corresponding to accession number NC_007761. A possible frameshift in the putative coding sequence of *Salmonella enterica* serovar Typhimurium LT2 *iolD* (accession numbers AAL23251 and AAL23252) was corrected by inserting one N after position 1342 in the sequence corresponding to accession number AE008908. The resulting translated products were used in this study. Multiple alignments were obtained using ClustalW (29) and manually corrected where necessary. Positions of doubtful homology or that introduced phylogenetic noise were removed by using Gblocks software (4). Phylogenetic reconstructions were performed by using maximum-likelihood inference as implemented in PHYML version 2.1b (14). We used the WAG substitution matrix (34), with a discrete gamma function with four categories plus invariant sites to account for substitution rate heterogeneity among sites and empirically estimated amino acid frequencies.

Nucleotide sequence accession number. The nucleotide sequence reported in this work has been deposited in the EMBL-GenBank under accession number EF382358.

RESULTS

Identification and analysis of the *L. casei* BL23 *iol* operon. Comparison of the incomplete genome sequence of *L. casei* strain BL23 (17) with that of the newly proposed type strain *L. casei* ATCC 334 (GenBank accession number CP000423.1) (22) revealed that BL23 carries numerous DNA insertions, which accounts for the difference in genome size between the two strains. One of these insertions consisted of a 12.8-kb-long DNA stretch located between a *B. subtilis yvgN* homologue and a putative gluconate utilization operon (Fig. 1). At both ends, the insert was flanked by a 19-bp sequence (CAACGGATTT CTAAGGCAA), whereas only a single copy of the 19-bp sequence was found in the ATCC 334 strain between *yvgN* and the gluconate operon (Fig. 1). This suggests that insertion in BL23 probably took place by recombination or that excision in ATCC 334 was caused by homologous recombination between the two direct repeats. Sequence analysis revealed that the inserted DNA probably contains genes involved in MI metabolism. The insertion starts with a gene encoding a DeoR-type transcriptional repressor (*iolR*). Ten genes were found to be transcribed in the opposite direction: *iolT* (MI transporter), *iolA* (malonate semialdehyde dehydrogenase), *iolB* (unknown function), *iolC* (2-deoxy-5-keto-D-gluconate kinase), *iolD* (encoding an enzyme resembling acetolactate synthase), *iolG1* and *iolG2* (encoding two MI dehydrogenases exhibiting similarity to each other), *iolE* (inosose dehydratase), *iolJ* (2-deoxy-5-keto-D-gluconic acid-6-phosphate dihydroxyacetone-phosphate lyase), and *iolK* (encodes a protein belonging to the tautomerase family). The phylogenetic analysis of *iolK* and homologous sequences (see Fig. S1 in the supplemental material) revealed a close relationship between the product of *iolK* and a malonate semialdehyde decarboxylase encoded by *Pseudomonas pavonaceae* (26). This result suggests that IolK also has malonate semialdehyde decarboxylase activity.

Hence, the *L. casei iol* gene cluster codes for all the enzymatic activities comprising the classical MI bacterial catabolic pathway, leading to the production of acetyl-coenzyme A (CoA) and dihydroxyacetone-phosphate (Fig. 2). However, the presence of a putative malonate semialdehyde decarboxylase (encoded by *iolK*) suggests that malonic semialdehyde, one of the products of the activity of the IolJ enzyme, can be metabolized through two different reactions. The first one would be catalyzed by the product of *iolA* with the formation of acetyl-CoA, whereas the second reaction would involve the product of *iolK* and lead to the formation of acetaldehyde (Fig. 2).

L. casei BL23 was able to ferment MI on MRS indicator agar plates containing 0.5% (wt/vol) of the polyol, whereas ATCC 334 was not. However, fermentation was observed only under aerobic conditions. Growing the cells in MRS liquid medium plus MI under static conditions did not result in acidification and gave only poor growth, whereas strong agitation resulted in acid production. MI fermentation was also tested on MRS indicator plates with seven other *L. casei* strains. Aside from BL23, three other strains (ATCC 4646, ATCC 11578, and 61BG) were able to produce acid from MI (Table 1). Amplification by PCR of *iolT* and *iolD* by using BL23-derived oligonucleotides was also possible in these MI-proficient *L. casei* strains (data not shown), suggesting that homologous *iol* genes are involved in MI catabolism in these strains.

To ascertain that the *iol* genes were responsible for the observed phenotype in BL23, disruptions of *iolT* (BL254), *iolD* (BL264), and *iolK* (BL265) were introduced in BL23 by integration of the pRV300 plasmid (20) carrying internal fragments of the respective genes. The *iolT* and *iolD* mutants failed to ferment MI, demonstrating that these genes are essential for MI catabolism. However, disruption of *iolK* had no significant effect on MI fermentation. A third mutant was constructed by

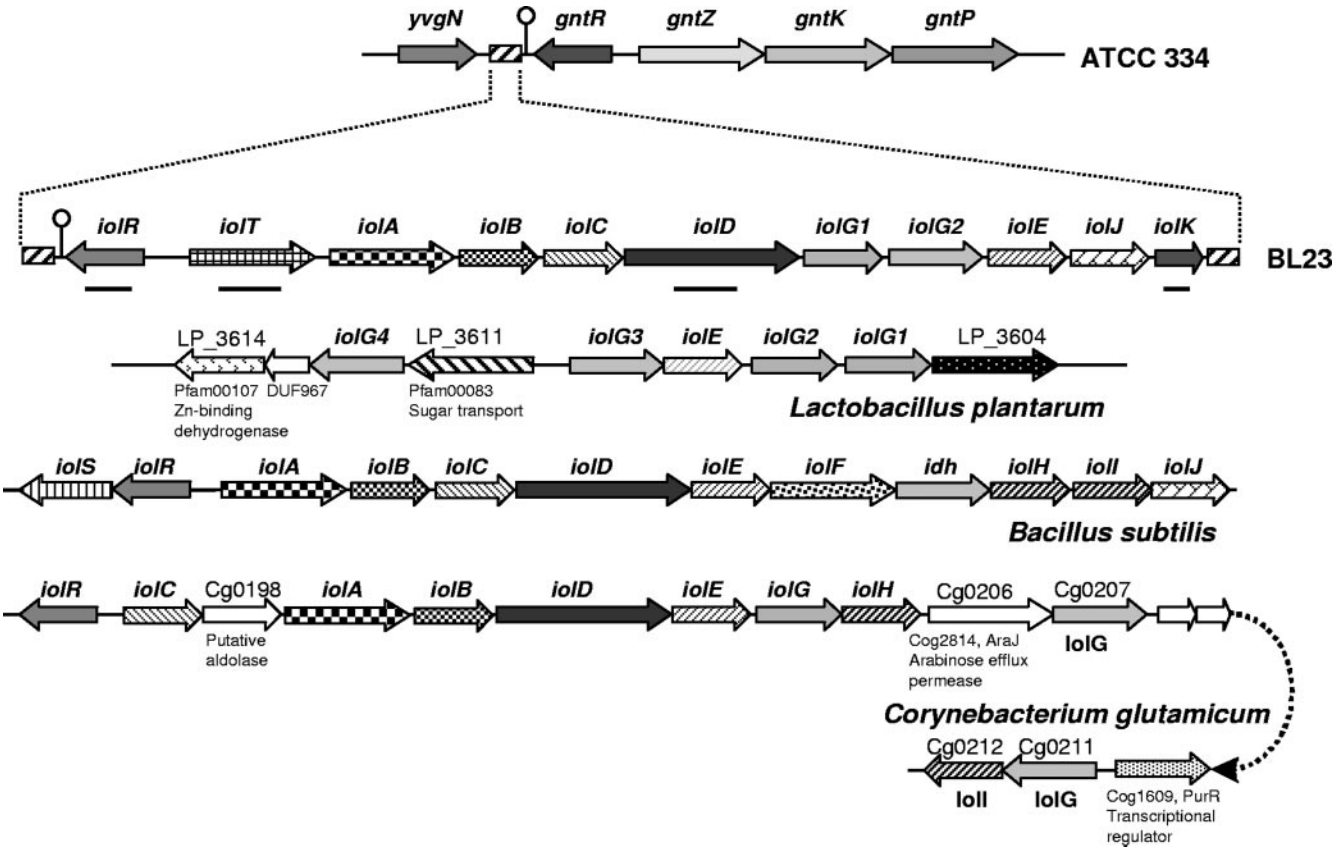

FIG. 1. Structural organization of the *L. casei* BL23 *iol* operon. The structure of the genes in the inserted DNA region of *L. casei* BL23 (12.8 kb) is presented in comparison to that of *L. casei* strain ATCC 334. The structures of the *L. plantarum*, *B. subtilis*, and *C. glutamicum iol* clusters are also shown. The positions of the 19-bp direct-repeat sequences flanking the inserted DNA are marked by boxes with diagonal stripes. Stem-loop structures in *L. casei* DNA represent putative transcriptional terminators. Horizontal solid bars represent DNA fragments used as probes and for gene disruption.

a double-crossover event, which introduced a frameshift at the level of *iolA* (BL276). In this strain, no plasmid vector remains integrated in the operon, and therefore minor effects on the expression of downstream *iol* genes are expected. This mutant was unable to produce acid from MI on agar plates, suggesting that blocking the conversion of malonic semialdehyde to acetyl-CoA prevents MI utilization.

Phylogenetic analysis of *iol* genes. Among all LAB genomes present in databases, including the recently published genomes of nine members of the *Lactobacillales* (22), homologues of *iol* genes were detected only in *L. plantarum*. The *iol* gene cluster harbored by *L. plantarum* shows significant differences from the *iol* cluster of *L. casei* BL23 (Fig. 1): *L. plantarum* has four *iolG* paralogues, *iolE*, and two genes encoding putative sugar permeases, while it lacks *iolA*, *iolB*, *iolC*, *iolD*, *iolJ*, and *iolK*. In agreement with this fact, *L. plantarum* NCIMB8826 and *L. plantarum* ATCC 14917 (type strain) were not able to ferment MI (data not shown).

In order to estimate whether *L. casei* recently acquired the *iol* gene cluster by horizontal gene transfer (HGT), the genome signatures were compared by calculating the genome signature dissimilarity scores (δ* values) between the *iol* gene cluster and the genome sequence of *L. casei* ATCC 334 (31). The δ* value of the *iol* cluster was 0.029. The percentage of genomic fragments of equal lengths exhibiting less genomic dissimilarity to the genome as a whole was 62.95%. This value indicates that the *iol* cluster does not differ significantly from the whole-genome composition (31). By contrast, similar analyses using the genome sequences of *Bacillus subtilis* 168, *Bacillus halodurans*, or *Clostridium perfringens* 13 instead of the *L. casei* BL23 genome sequence gave significant differences (percentages of less-dissimilar fragments were 88.99%, 84.7%, and 100%, respectively).

In order to gain insight into the origin and evolution of the *L. casei iol* cluster, a phylogenetic analysis of *iolD*, *iolE*, and *iolG* homologues was carried out. Both *iolE* and *iolD* genes rendered phylogenetic reconstructions with high support values even in deep nodes (Fig. 3 and see Fig. S2 in the supplemental material). In contrast, the *iolG* phylogenetic tree was poorly resolved (see Fig S3 in the supplemental material). The fact that many strains contain several *iolG* paralogues may partly account for this result. The phylogenetic trees show that none of the three genes studied grouped according to the order of descent based on phylogenetic markers such as 16S rRNA. Furthermore, the congruence among the three trees is also low. HGT possibly accounts for many of the observed discrepancies. For example, the location of the *iolE* and *iolG* genes of *Salmonella enterica* serovar Typhimurium LT2 close to their

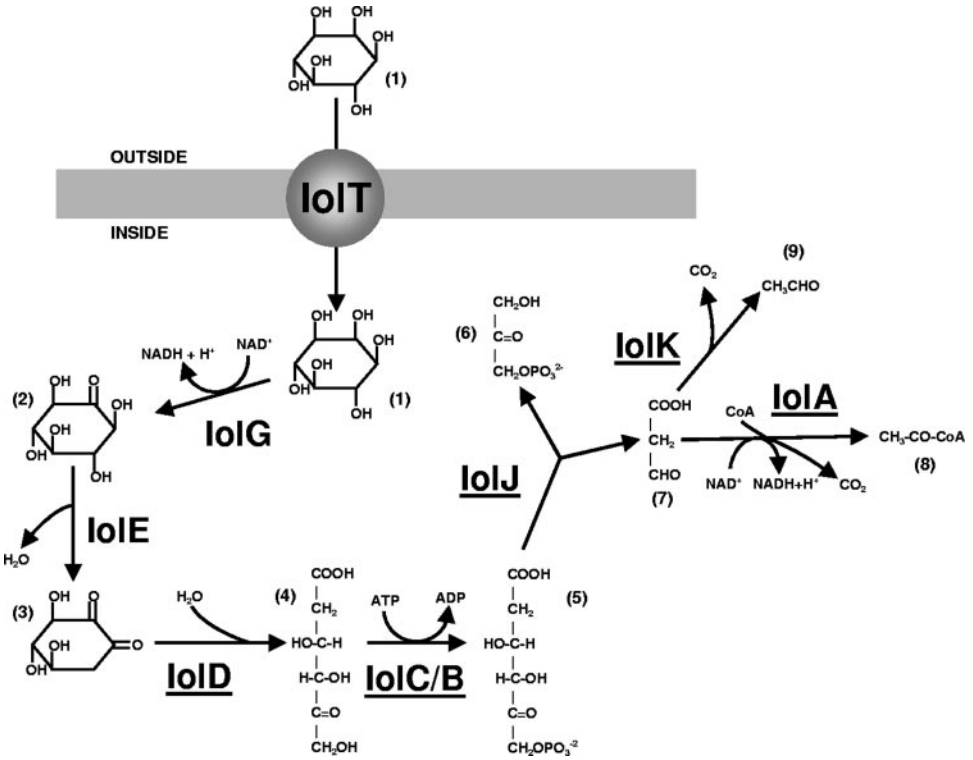

FIG. 2. Proposed MI catabolic pathway in *L. casei* BL23. For the *B. subtilis iol* genes, the functions of only *iolT*, *iolG*, and *iolE* have been experimentally elucidated. The functions of the other genes are assigned based on sequence homology, and their gene products are underlined. The function of *iolB* is unknown, but in *Legionella pneumophila*, *iolB* is fused to *iolC* (accession number YP_123940), which is probably coding for a 2-deoxy-5-keto-D-gluconate kinase. In *L. casei*, *iolK* (encoding a probable malonic semialdehyde decarboxylase) might define an alternative pathway for the conversion of malonic semialdehyde to acetaldehyde. The metabolites are numbered as follows: (1), MI; (2), inosose; (3), D-2,3-diketo-4-deoxy-epi-inositol; (4), 2-deoxy-5-keto-D-gluconate; (5), 2-deoxy-5-keto-D-gluconate-6-phosphate; (6), dihydroxyacetone-phosphate; (7), malonic semialdehyde; (8) acetyl-CoA; (9), acetaldehyde.

homologues from *L. plantarum* in clusters of genes of the *Firmicutes* indicates that *iolE* and both *iolG* paralogues were probably acquired from *L. plantarum* (Fig. 3 and see Fig. S2 in the supplemental material). In contrast, *Salmonella* serovar Typhimurium *iolD* is closely related to genes from other members of the *Gammaproteobacteria* (see Fig. S3 in the supplemental material). This result, together with the great variation observed in both gene content and order in *iol* clusters (see Fig. S4 in supplemental material), makes it very difficult to track down the origin of these clusters.

The positions of the *L. casei* genes analyzed here show a close relationship with those of their counterparts from the *Firmicutes*. However, significant differences can be observed from one gene to another: *iolD* appears as a basal branch of a cluster with strong support which contains genes from *Bacillus* and *Listeria* (see Fig. S3 in the supplemental material). In contrast, *L. casei iolE* occupies an uncertain position in a cluster which includes all genes from the *Firmicutes* as well as genes from the *Gammaproteobacteria*, one of the *iolE* genes present in *Rhizobium etli*, and two *iolE* homologues from the *Cyanobacteria* (Fig. 3), whereas *L. plantarum iolE* is closely related to its counterparts harbored by *Bacillus clausii*, *B. halodurans*, *Bacillus licheniformis*, and *B. subtilis*. Finally, the two *L. casei iolG* genes are only distantly related to each other (see Fig. S2 in the supplemental material). Therefore, these genes did not originate from a recent duplication event. Gene *iolG1* is closely related to *idh* genes from *B. subtilis* and other bacilli as well as to *iolG2* from *L. plantarum*. On the other hand, *L. casei iolG2* is related to genes harbored by clostridia, *B. cereus*, and *B. halodurans* and more distantly related to *L. plantarum iolG1* and one of the *iolG* homologues harbored by *Mycoplasma hyopneumoniae* (*mioJ1*).

Transcription of *iol* genes is induced by MI and repressed by IolR. Northern blot analysis showed that *iol* genes were transcribed only in the presence of MI and that their transcription was repressed by the presence of glucose (Fig. 4A). Similar results were obtained when either *iolT*, *iolD*, or *iolK* probes were used (not shown). On the contrary, Northern blot analysis with an *iolR* probe showed that the *iolR* messenger was present at similar levels irrespective of the carbon source used for growth (Fig. 4B). The size of its transcript (1 kb) was in agreement with an mRNA covering the whole *iolR* gene and expanding up to the position of a putative *rho*-independent terminator (−23.2 kcal/mol) placed at the end of the insertion point (Fig. 1). Except for *iolR*, no putative transcriptional terminator was found in the inserted region, and some of the stop codons of the *iol* genes overlapped start codons or Shine-Dalgarno sequences of the following genes, suggesting translational coupling. This indicates that the *iolTABCDG1G2EJK* cluster is transcribed as a long mRNA (11 kb) that terminates at a stem-loop structure (−19.5 kcal/mol) located 36 bp downstream from the insertion point (Fig. 1). This stem-loop could

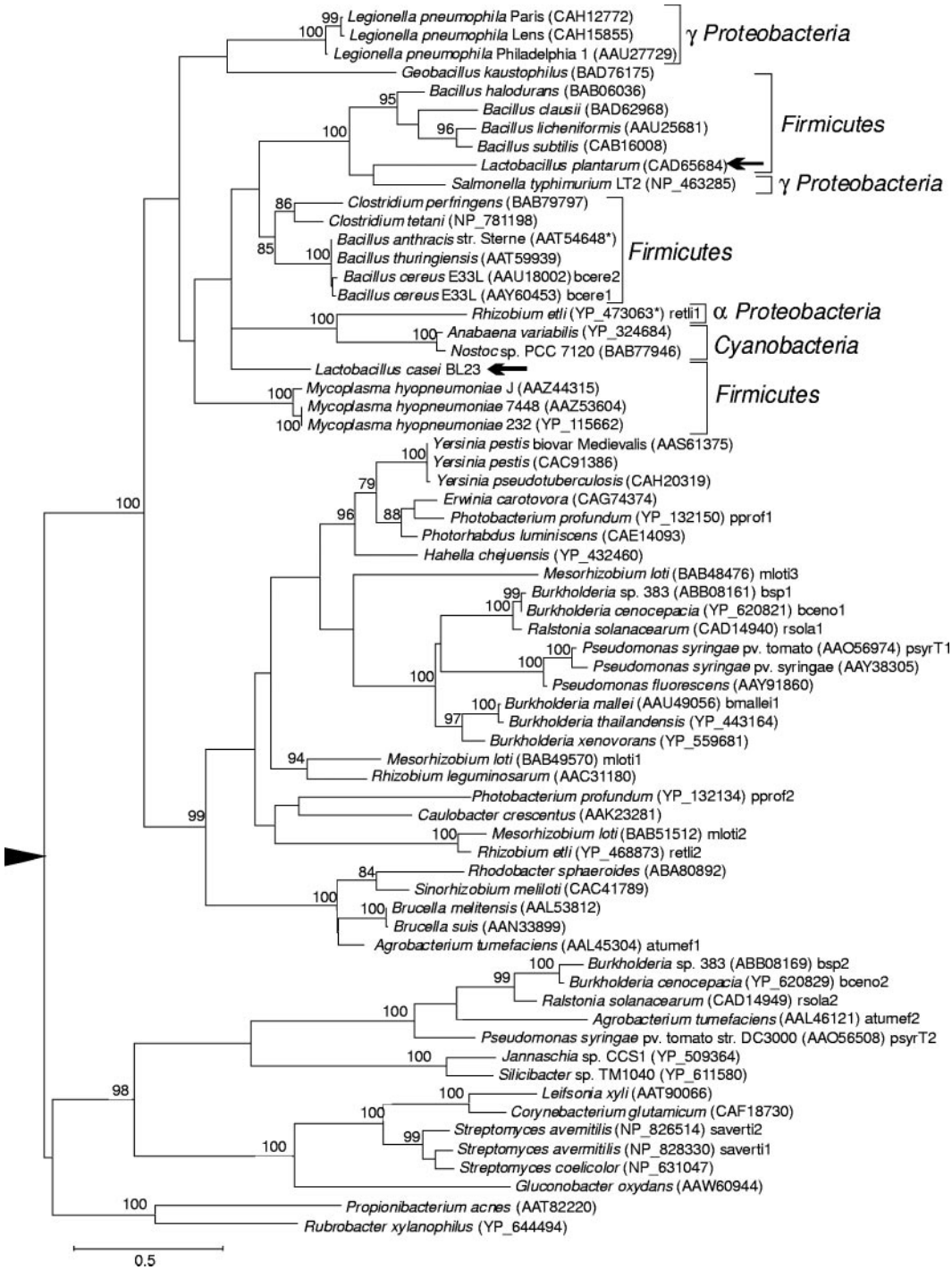

FIG. 3. Phylogenetic reconstruction of *iolE* genes. The tree is arbitrarily rooted as indicated by the black triangle. Asterisks indicate sequences that have been modified as indicated in Materials and Methods. Paralogous genes harbored by the same organism are indicated by labels following the accession numbers. The arrows indicate the positions of genes harbored by lactobacilli.

also serve as a *rho*-independent terminator for the *gntR* gene, which is transcribed in the opposite direction in both the BL23 and ATCC 334 strains. Northern blot analysis did not allow detection of the putative long *iol* mRNA, and most of the signal was at the level of ribosomal RNAs (Fig. 4A and 4C), indicating comigration or degradation of the RNA, a problem usually found when analyzing long *Lactobacillus* transcripts (35).

The *iolR* gene was inactivated by the integration of a suicide plasmid, giving strain BL255. Transcription of the other *iol* genes in this mutant occurred independently of the presence of MI, thus supporting the concept that IolR is a transcriptional

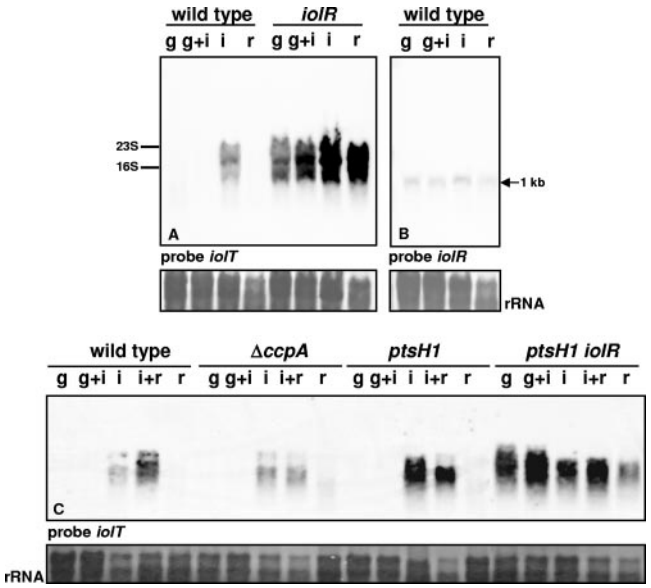

FIG. 4. Northern blot analysis of the transcription of *iol* genes in *L. casei* BL23. RNA was isolated from strains grown on glucose (g), MI (i), glucose plus MI (g+i), MI plus ribose (i+r), and ribose (r) and hybridized with probes covering *iolR* and *iolT*. The culture medium for RNA isolation was inoculated with cells pregrown on MI. A stain of the rRNA in each membrane is shown below the blots. Strains used were BL23 (wild type), BL121 (*ptsH1*), BL190 (Δ*ccpA*), BL255 (*iolR*), and BL256 (*ptsH1 iolR*).

repressor (Fig. 4A). In this mutant, the presence of glucose in the culture medium caused a decrease in messenger levels, indicating that transcription was also regulated by CCR (Fig. 4A).

Unfortunately, no quantitative measurements of MI dehydrogenase activity were possible, owing to the high NADH oxidase activity present in *L. casei* crude extracts. In contrast to that in *B. subtilis* strains (6), MI oxidation was not measurable in crude extracts prepared from BL23 cells grown in the presence of MI. Nevertheless, MI dehydrogenase activity was clearly measurable in the *iolR* mutant grown either on ribose or ribose plus inositol (enzyme activities within the first 30 seconds were in the order of 20 nmol of product formed per min and mg of protein but rapidly leveled off). Under repressing conditions, when the growth medium was supplemented with MI and glucose, MI dehydrogenase activity was no longer measurable, not even during the first 30 seconds. The enzymatic assays at least qualitatively confirmed the results obtained by Northern blot analyses.

Regulation of *iol* genes by the PTS/CcpA pathway. A sequence resembling a *cre* site (AGTAAGCGCTTAAT), the binding site of the CcpA/P-Ser-HPr complex (30), was found in the *iolT* promoter region, suggesting that CCR of the *iol* operon was mediated via the PTS/CcpA pathway (5). To prove this hypothesis, RNA was isolated from strains BL190 (*ccpA* deletion mutant) and BL121 (a strain carrying the *ptsH1* mutation and therefore producing HPr, which cannot be converted to P-Ser-HPr, the CcpA corepressor, as the Ser-46 residue is replaced with Ala) (32). None of these mutations provided relief from the glucose repression of *iol* genes, as no expression was detected in a medium containing glucose plus MI (data not shown). To avoid the possibility that the *iol* genes were not yet induced at the moment of RNA isolation due to the rapid growth provided by glucose, similar experiments were repeated by inoculating liquid medium with cells which had previously been grown on MI, a condition where the *iol* genes should be fully induced. Again, no transcription was detected in the presence of glucose in any of the CCR mutants, whereas the addition of ribose (nonrepressing sugar) allowed the normal induction of the *iol* genes (Fig. 4C). Glucose repression in a *ccpA* or *ptsH1* background might be mediated by an inducer exclusion effect on MI that would act at the level of IolR (the presence of glucose blocks MI transport and hence prevents the induction of the *iol* genes). In order to clearly demonstrate the involvement of the PTS/CcpA pathway in the CCR of *iol* genes, the *iolR* gene was inactivated in a *ptsH1* background, providing the BL256 (*ptsH1 iolR*) double mutant. A complete relief of glucose repression was found in this strain (Fig. 4C). Unfortunately, we could not study the CCR effect in a *ccpA iolR* double mutant because, for unknown reasons, this strain could not be constructed.

DISCUSSION

We showed that, in contrast to ATCC 334, *L. casei* BL23 carries a complete set of genes for the metabolism of MI. However, *L. casei* BL23 was able to metabolize MI only under aerobic conditions. This is in agreement with the finding that the fermentation of other hexitols (sorbitol, mannitol) was improved when the *L. casei* cells were grown under aeration (M. J. Yebra, unpublished observations). In addition, an *L. casei* mutant with impaired NADH oxidation (lactate dehydrogenase-deficient mutant) grew significantly faster when the culture was aerated (33). The degradation of one MI molecule produces two NADH equivalents in the course of the first enzymatic reactions, leading to the formation of acetyl-CoA and dihydroxyacetone phosphate (the dehydrogenase step catalyzed by IolG and the conversion of malonic semialdehyde to acetyl-CoA catalyzed by IolA). Therefore, an elevated NADH/NAD^+ ratio was possibly responsible for the poor growth on MI under static (anaerobic) conditions. By contrast, growth under aeration can be enhanced by the activity of O_2-dependent NADH oxidases that could reoxidize excess NADH. Although there is no experimental evidence of O_2-dependent NADH oxidase activity in *L. casei*, the inspection of the available genome sequence of *L. casei* BL23 shows that this organism encodes at least three putative NADH oxidases (data not shown). Furthermore, there is evidence of the involvement of NADH oxidases in NAD^+ regeneration in other lactobacilli (13, 23).

The scarceness of *iol* clusters in LAB, together with the relative similarity of the *L. casei iol* cluster to *iol* clusters harbored by other *Bacillus* species (Fig. 1), suggested that *L. casei* might have acquired *iol* genes from *Bacillus* by HGT. Nevertheless, although phylogenetic analyses place the origin of the *L. casei iol* cluster in the *Firmicutes*, our results do not support the idea that *L. casei* acquired the *iol* cluster by a recent HGT from one of the organisms discussed here. First, the calculation of the δ^* values showed that the *L. casei iol* cluster does not significantly differ from the whole-genome composition, thus arguing against a recent acquisition of the *iol* cluster by HGT.

This result also suggests that the absence of the *iol* cluster in *L. casei* ATCC 334 is due to a deletion in this strain. Furthermore, the phylogenetic analyses suggest that the *iol* clusters harbored by *L. casei* and *L. plantarum* either do not come from a common ancestor or have sustained major modifications during the evolutionary history of these species. These changes would at least include the loss and/or gain of genes and nonorthologous displacements (*iolE*, for example). The data presently available do not allow the determination of which possibility is more probable. In summary, our results indicate that the *iol* cluster was acquired by *L. casei* from a *Firmicutes* donor in a relatively early stage of the evolution of this species, and it was subsequently lost in some strains. This is in agreement with the current view of the evolution of the *Lactobacillales*, in which the adaptation of LAB to nutrient-rich habitats was accompanied by significant gene losses and the emergence of new genes via duplication and HGT (22).

Despite its similarity to that of *B. subtilis*, the *L. casei iol* operon contains distinct features related to gene content and regulation. (i) In *L. casei*, a putative malonic semialdehyde decarboxylase gene is transcribed with the other *iol* genes. The gene encoding this enzyme was first described in *Pseudomonas pavonaceae* and was clustered in an operon involved in the degradation of the xenobiotic *trans*-1,3-dichloropropene (26). The presence of this gene in *L. casei* suggests an alternative pathway for the metabolism of malonic semialdehyde different from the classical oxidative decarboxylation catalyzed by the product of *iolA* (Fig. 2). The *B. subtilis* genome contains two *iolK* homologues (*yrdN* and *yodA*) which are not clustered with *iol* genes. The phylogenetic analysis suggests that the products of these genes also have malonate semialdehyde decarboxylase activity. However, it remains unknown whether these genes are involved in MI catabolism in *B. subtilis*. Inactivation of *iolK* and *iolA* showed that, while *iolA* was indispensable for MI fermentation, a mutation in *iolK* caused no apparent phenotype under our laboratory conditions. The physiological relevance of each gene is not known, but it can be postulated that each pathway could make a different contribution to the catabolism of malonic semialdehyde depending on the growth conditions (anaerobic conditions might favor the IolK pathway). (ii) Three genes belonging to the *B. subtilis* MI regulon are absent from *L. casei* BL23. *L. casei* does not possess *iolH*, *iolI*, or *iolS* homologues. The functions of *iolH* and *iolS* are not known, but *iolI* encodes an inosose isomerase, which converts inosose to 1-keto-D-*chiro*-inositol (38). In any case, none of these genes is essential for MI utilization. Furthermore, *B. subtilis* has two MI transporters encoded by *iolF* and *iolT* (39). The transporter encoded by *iolT* has a higher affinity, but a complete loss of MI utilization is obtained only upon the mutation of both genes. Similarly, two MI transporters which exhibited comparable kinetic parameters (IolT1 and IolT2) have been identified in *C. glutamicum* (19). It is not known whether *iolT* is the only gene involved in MI uptake in *L. casei* BL23. The disruption of *iolT* renders an MI-negative phenotype, but the integration of a nonreplicative plasmid at this locus (strain BL254) probably leads to polar effects on the other *iol* genes (except *iolR*). In addition, *L. casei* carries two different inositol dehydrogenase-encoding genes (*iolG1* and *iolG2*). Phylogenetic analysis does not support the idea that duplication would be the cause for the two *iolG* genes. It is not known at this stage whether they have redundant functions or possess different substrate affinities toward inositol isomers or related molecules. (iii) The *B. subtilis iol* regulon is repressed by IolR, and it is subject to CCR regulation mediated by CcpA. Both mechanisms result in a repression ratio (i.e., of the expression on MI to the expression on MI plus glucose) of greater than 100 (6, 11, 24). *iolR* transcription in *B. subtilis* and *C. perfringens* is induced by MI (16, 40), whereas its transcription in *L. casei* was constitutive. Our results suggest that IolR and CcpA act synergistically to regulate *iol* genes in *L. casei*. However, regulation in *L. casei* occurs mainly through IolR, whereas CcpA-dependent repression plays a minor role. A *cre* sequence is present in the promoter region, but the CCR effect triggered by CcpA was weak and became visible only after the comparison of the *iolR* and *iolR ptsH1* mutants. By contrast, Deutscher et al. (6) reported that a *ccpA* mutation completely abolished the glucose repression of *iol* genes in *B. subtilis*. However, residual repression (about 10% to that of the wild type) was detected in a *ccpA* background by Yoshida et al. (37). The authors claimed that this discrepancy might be due to the different compositions of the growth media and pointed to inducer exclusion as the phenomenon responsible for the residual CcpA-independent glucose repression. Inducer exclusion might also be responsible for glucose repression in the *L. casei ccpA* or *ptsH1* mutants. This regulatory mechanism has been studied in LAB for the transport of maltose, ribose, and galactose (7, 32) and shown to be dependent on P-Ser-HPr, which interacts with and blocks non-PTS transporters. Because the repression of *iol* genes is still operative in an *L. casei ptsH1* mutant unable to form P-Ser-HPr, a P-Ser-HPr-independent mechanism needs to be operative to explain the regulation of the *iol* operon in *L. casei*.

Despite the fact that a large number of putative *iol* genes are found in genome sequences, there is only limited information available about their functionality, and only a few of them have been characterized. In this work, we identified and analyzed an *iol* gene cluster of *L. casei* BL23 and demonstrated its implication in MI utilization in this bacterium. The physiological role of the *L. casei* BL23 MI pathway is probably not related to phytate utilization, as no phytase-encoding gene has been found in ATCC 334 or BL23 genomes. Phytates have been reported to enhance the growth of certain lactobacilli, but this result might be a consequence of the increased availability of Mg^{2+} or Fe^{3+} generated by phosphate removal from phytates (21, 25). *L. casei* and lactobacilli in general thrive in complex niches characterized by the availability of numerous fermentable sugars, such as the gastrointestinal tract and decaying plant material. The *iol* genes may offer some advantage to *L. casei* BL23 in the latter niche, where MI is abundant.

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